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Correlation of the Quality of Sexed-Spermatozoa of Polled Bali Bull and Protein Molecular Weight

A. T. Nurgina [1], S. Baco [2], H. Sonjaya [2], R. Malaka [2], S. Gustina [2], E. Damayanti [2,3], E. M. Kaiin [3], T. Maulana [3], M. Gunawan [3] and H. Hasbi [2 *]

[1]Faculty of Animal Science, Graduate School of Hasanuddin University Jl. Perintis Kemerdekaan Km.10, Makassar, Indonesia 90245
[2]Department of Animal Production, Faculty of Animal Science, Hasanuddin University Jl. Perintis Kemerdekaan Km.10, Makassar, Indonesia 90245
[3]Research Center for Applied Zoology, National Research and Innovation Agency Jl. Raya Bogor-Jakarta Km.46, Cibinong, Indonesia 16911
***Corresponding author:** hasbi_fapetunhas@yahoo.com

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ABSTRACT

Polled Bali cattle are a species of Bali cattle that are naturally hornless and whose productivity can be increased by artificial insemination (AI) using sexing semen so that the sex of the calf can be controlled according to the breeder's wishes. This research used sexed spermatozoa from polled Bali cattle originating from three bulls. Motility and kinematics of spermatozoa were evaluated by computer-assisted semen analysis (CASA), viability and abnormalities were evaluated by eosin-nigrosine staining, intact plasma membrane (IPM) by HOS Test, acrosome status by FITC-PNA-PI, DNA fragmentation by AO Fluorescence, and molecular weight by SDS-PAGE. The results showed that motility, viability, and IPM between the upper and lower fractions were significantly different ($P<0.05$), while abnormalities, acrosome integrity, and DNA fragmentation were not significantly different ($P>0.05$). The results of linearity analysis showed that several protein bands in X and Y spermatozoa in the range 12-70 kDa had a positive correlation between protein molecular weight and motility, viability, abnormalities and IPM but negatively correlated with acrosome integrity and DNA fragmentation. The decrease in spermatozoa quality was thought to be caused by physical injury to spermatozoa due to the sexing process. It can be concluded that the protein bands of X and Y spermatozoa of polled Bali bulls were positively correlated with motility, viability, abnormalities and IPM, but negatively correlated with acrosome integrity and DNA fragmentation.

Key words: Bali cattle, Polled bull, Protein molecular weight, Sexing spermatozoa, Quality of spermatozoa

INTRODUCTION

Bali cattle are local livestock that are widely developed in South Sulawesi (Talib 2002). Regarding the development of Bali cattle, a species of Bali cattle was found to be naturally hornless (polled). This species originates from a cross between Bali cattle (*Bos sondaicus*) and Brahman Cross (BX) cattle, which is a cross between Brahman cattle and Hereford cattle (Baco et al. 2020). The increasing of population of polled Bali cattle can be made by implementing Artificial Insemination (AI) by optimizing superior bulls with the advantage of easy maintenance and minimal accidents for farmers and livestock (Fesrianti et al. 2023).

Optimizing the application of AI can be supported by the use of spermatozoa resulting from sexing, which has potential to increase profitability, accelerate livestock expansion, and increase livestock populations (Pahmeyer and Britz 2020). The success of AI is greatly influenced by the quality of spermatozoa (Manehat et al. 2021). Spermatozoa characteristics can be assessed based on motility, kinematics, morphology, viability, acrosome integrity, plasma membrane integrity, and DNA integrity (Bernecic et al. 2021).

Molecular components in spermatozoa have an essential role in bull fertility (Moura and Memili 2016). Evaluation of spermatozoa biomarker profiles can be done by identifying proteins to determine the physiology and

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molecular anomalies of spermatozoa (Tanga et al. 2021) because sperm proteins play a role in the fertilization process, cell activation, and embryo development (Moura and Memili 2016). Identification of sexing sperm proteins has been carried out in cattle (De Canio et al. 2014), Holstein cattle (Chen et al. 2012) and Nelore breed bulls (Scott et al. 2018). Several studies have reported protein content in spermatozoa that acts as a marker of fertility, such as the TUBA8 protein detected in sexing spermatozoa (De Canio et al. 2014). It plays a role in spermatogenesis with a molecular weight of 55kDa (Diggle et al. 2017). A molecular weight of 31kDa was detected as an HBP protein (Ramteke et al. 2014). This research needs to be carried out to determine the correlation between the quality and protein molecular weight of spermatozoa of polled Bali bull resulting from sexing process.

MATERIALS AND METHODS

Animals and ethical clearance

This research used three polled Bali bulls aged 5-8 years kept at the Technical Services Unit of the Center for Artificial Insemination and Semen Production (SU-CAISP) Pucak, Maros, Indonesia with the raising bulls system according to the existing Standard Operating Procedures (SOP). Feeding was done twice a day (morning and evening) with fresh forage 10% of body weight and concentrate 2kg/head/day. The Hasanuddin University Animal Ethics Commission approved this animal model and research design with certificate number 302/ UN4.6.4.5.31/PP36/2021.

Semen collection and evaluation

Semen of polled Bali bulls was collected using an artificial vagina. Macroscopic and microscopic evaluation was done according to the SOP for semen evaluation at SU-CAISP.

Sexing of semen

Sexing of semen was done using the Bovine Serum Albumin (BSA) gradient method with a concentration of 5% (upper fraction) and 10% (lower fraction) following the procedure by Kaiin and Gunawan (2017) and frozen based on SOP at SU-CAISP.

Motility, viability, abnormality, intact plasma membrane, progresif motility, Kinematic, acrosome integrity, DNA fragmentation analysis

Thawing was carried out in a water bath at 37°C for 30s. The motility and kinematics of spermatozoa were evaluated by computer-assisted semen analysis (CASA) with the Sperm Vision Program application (Minitub Tiefenbach, Germany) following the procedure by Maulana et al. (2022). The parameters observed include total motility (%), progressive motility (%), velocity average path (VAP: µm/second), velocity curvilinear (VCL: µm/second), velocity straight linear (VSL: µm/second), straightness (STR: VSL/VAP), linearity of forward progression (LIN: VSL/VCL), average lateral head displacement (ALH: µm), wobble (WOB: VAP/VCL) and beat cross frequency (BCF: Hz).

Viability and abnormalities of spermatozoa were observed by Eosin-Nigrosin staining according to Handayani et al. (2021) and observed under a light microscope (Olympus CX23, Japan) with a magnification of 400×. Viability and abnormalities of sperm were identified based on assessment, according to Auger et al. (2016). Intact plasma membranes (IPM) were observed using the Hypo-Osmotic Swelling (HOS) Test method according to Allouche et al. (2017) and samples were observed under an Olympus CX23 microscope with 200× magnification at five viewing angles with an assessment based on research by Ramu and Jeyendran (2013).

Evaluation of acrosome status was carried out using the Fluorescein isothiocyanate (FITC) method, according to Fannessia et al. (2015). The samples were observed under a fluorescence microscope with a wavelength of 380-420nm. Spermatozoa with intact acrosomes are green, and spermatozoa with incomplete acrosomes are not green/red.

DNA fragmentation was observed by acridine orange (AO) staining, according to Handayani et al. (2021). The samples were observed under a fluorescence microscope with a wavelength of 450-490nm at 400x magnification in 10 fields of view. Fragmented spermatozoa were orange, and unfragmented spermatozoa were green.

Evaluation of protein molecular weight by SDS-PAGE

Evaluation of the molecular weight of spermatozoa proteins was carried out by SDS-PAGE according to Baharun et al. (2023) with some modifications. Protein molecular weight was analyzed based on molecular weight (MW), which was presented by the presence of protein bands. The differential intensity of each protein band was assessed by performing ratio analysis with ImageJ software.

Statistical Analysis

The data of semen quality were analyzed using a completely randomized design, one-way ANOVA and between fractions T-tested in the IBM SPSS Statistics for Windows program, Version 16.0 (IBM Corp., NY, USA) was applied to the different data groups. The correlation between sperm quality and protein molecular weight of sperm sexing was analyzed with Pearson's correlation.

RESULTS

Quality of sexed-spermatozoa

Spermatozoa motility in upper fraction was significantly higher ($P<0.05$) in bull 11745 compared to both 11437 and 11746, but 11437 and 11746 were not different ($P>0.05$). Sperm motility of 11437 and 11746 bull was significantly higher in the lower fraction than in the upper fraction ($P<0.05$). The viability of spermatozoa in the upper fraction was significantly higher ($P<0.05$) on bull 11745 compared to both 11437 and 11746; but the viability of bull 11746 was significantly higher compared to 11437 one. Meanwhile, the lower fraction did not differ ($P>0.05$). The sperm viability of bulls 11437 and 11746 was significantly higher ($P<0.05$) in the lower fraction compared to the upper fraction. Intact Plasma Membrane of spermatozoa in the upper and lower fractions were significantly higher ($P<0.05$) on bull 11745 compared to both 11437 and 11746. Abnormality parameter, acrosome integrity and DNA fragmentation in both between fractions and bulls, were not statistically different ($P>0.05$) (Table 1).

Table 1: The Sexed-Spermatozoa Quality of Polled Bali Bulls

Parameters (%)	ID Bulls					
	11437		11745		11746	
	Upper faction	Lower fraction	Upper faction	Lower fraction	Upper faction	Lower fraction
Motility	31.99±5.56aA	46.92±1.31B	43.59±2.05b	44.82±2.12	33.02±2.93aA	45.17±2.31B
Viability	50.98±0.54aA	60.50±2.13B	60.80±0.79b	61.61±2.02	57.95±0.93cA	61.98±2.33B
Abnormalities	11.81±1.56	15.25±3.35	15.89±4.66	16.95±4.01	14.46±3.81	16.20±4.32
Intact Plasma Membrane	55.28±1.45a	57.61±2.65a	57.40±0.66bA	64.18±0.91bB	55.16±1.28aA	59.83±1.88aB
Acrosome Integrity	71.74±13.24	86.51±1.03	74.79±5.60	83.90±1.97	74.03±2.81	87.45±10.27
DNA Fragmentation	98.14±0.00	98.46±0.77	97.00±2.12	98.50±0.71	98.25±0.35	99.02±0.00

Values (Mean±SD) bearing small letters show the difference between upper or lower fractions on different bulls in the same row, while capital letters show the differences between upper and lower fractions on the same bull in the same row.

Table 2: Computer-Assisted Semen Analysis (CASA) Movement Patterns of Sexed-Spermatozoa of Polled Bali Bulls

Parameters	ID Bulls (Mean±SD)					
	11437		11745		11746	
	Upper faction	Lower fraction	Upper faction	Lower fraction	Upper faction	Lower fraction
P.Mot	24.65±2.65aA	41.88±3.26B	37.75±4.52b	39.52±4.23	25.59±6.72Aa	41.71±1.94B
VAP (µm/s)	68.41±5.60a	84.74±20.49	75.50±4.02a	8327±9.15	56.89±3.74bA	69.84±5.40B
VCL (µm/s)	99.09±16.84ab	117.03±16.11	109.72±6.41b	116.07±6.12	83.36±6.55aA	103.75±12.78B
VSL (µm/s)	53.89±7.23b	71.13±23.74	62.46±5.75b	69.38±10.59	40.71±4.74aA	53.11±4.95B
ALH (µm)	2.91±0.97	3.70±0.89	3.60±0.35	3.01±0.38	3.34±0.72	3.29±0.38
STR (%)	0.78±0.07ab	0.82±0.08	0.82±0.04b	0.83±0.05	0.71±0.04a	0.76±0.03
LIN (%)	0.55±0.09	0.59±0.13	0.57±0.04	0.59±0.07	0.49±0.05	0.51±0.02
WOB (%)	0.69±0.07	0.71±0.09	0.69±0.02	0.71± 0.05	0.68±0.05	0.68±0.04
BCF (Hz)	29.30±1.24b	30.47±3.43b	31.59±3.04b	30.06±1.83	24.19±1.73Aa	28.14±1.33B

Values (Mean±SD) bearing small letters show the difference between upper or lower fractions on different bulls in the same row, while capital letters show the differences between upper and lower fractions on the same bull in the same row. P.Mot (Progressive motility), VAP (velocity average path), VCL (velocity curvilinear), VSL (velocity straight-line), ALH (amplitude of lateral head displacement), STR (straightness), LIN (linearity), WOB (wobble) dan BCF (beat cross frequency).

The progressive motility of spermatozoa in the upper fraction was significantly higher (P<0.05) on bull 11745 compared to 11437 and 11746. The progressive motility of spermatozoa on bulls 11437 and 11746 was significantly higher in the lower fraction compared to the upper fraction (P<0.05). However, on bull 11745, the upper and lower fractions were not significantly different (P<0.05). VAP, VSL, and BCF of spermatozoa in the upper fraction were significantly lower (P<0.05) on bull 11746 compared to bulls 11437 and 11745, but 11437 and 11745 were not different (P>0.05). VCL and STR of sper3matozoa in the upper fraction were significantly higher (P<0.05) on bull 11745 compared to 11746.

VAP, VCL, VSL, and BCF of spermatozoa of bull 11746 were significantly higher in the lower fraction compared to the upper fraction (P<0.05), but on bulls 11437 and 11745 were not significantly different (P<0.05) between the upper and lower fractions. Meanwhile, the ALH, LIN, and WOB parameters between factions and bulls were not significantly different (P>0.05) (Table 2).

The molecular weight of spermatozoa proteins

The expression of the amount of protein in the lower and upper fractions between the three bulls has difference (Fig. 1). The results of linearity analysis showed that the protein molecular weight has a positive correlation with sexed-spermatozoa quality of polled Bali bulls (Fig. 2) on the sperm motility (r=0.087), viability (r=0.046), abnormalities (r=0.049), and IPM (r=0.021), but has a negative correlation on acrosome integrity (r=-0.006) and DNA fragmentation (r=-0.008).

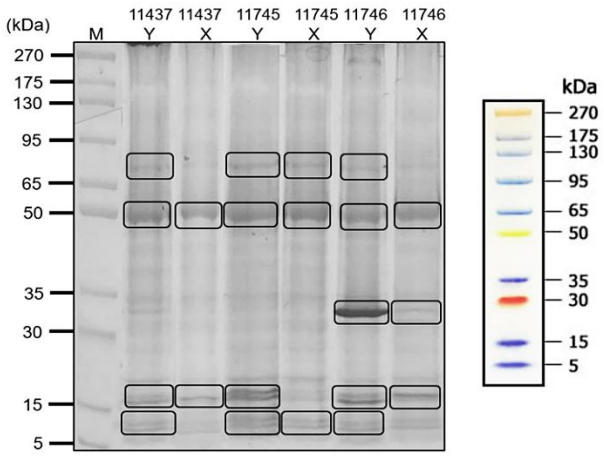

Fig. 1: Protein molecular weight of sexed spermatozoa of polled Bali bulls.

DISCUSSION

The motility of sexed sperm from polled Bali bull in this study was found to be lower than that previously reported by Anwar et al. (2019), the motility of spermatozoa resulting from sexing with upper and lower fractions of BSA medium is the same, around 57-72%. The low motility of spermatozoa in the fraction is thought to be due to the long incubation time so that spermatozoa can penetrate the medium of the lower fraction quickly, and in the upper fraction, only spermatozoa with low motility. The semen is still suitable for use (Muhammad et al. 2016), semen with a PTM quality of 20-30% produces a pregnancy of around 85-95% with AI deposition in position 4+.

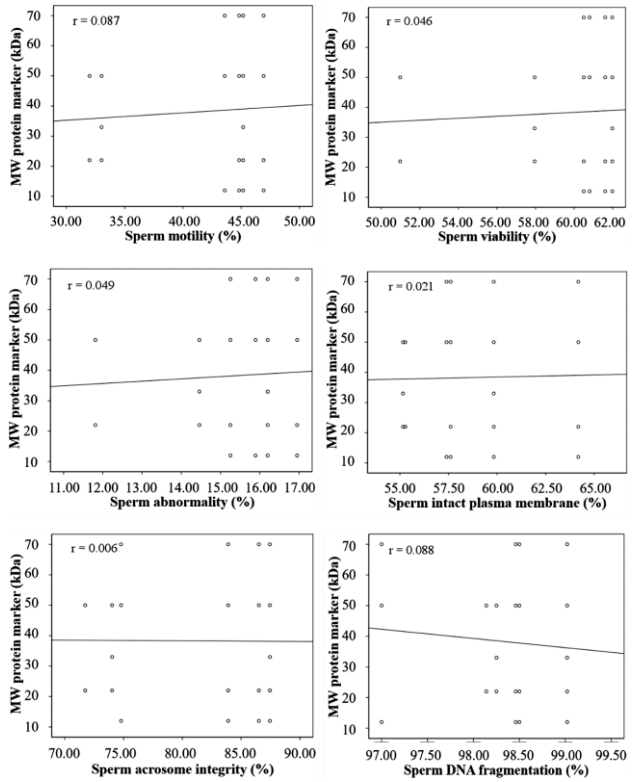

Fig. 2: Correlation between motility, viability, abnormalities, intact plasma membrane, acrosome integrity and DNA fragmentation with protein molecular weight of sexed-spermatozoa of polled Bali bulls.

Spermatozoa viability analysis is carried out with the aim of determining whether non-motile spermatozoa are alive or not (Samplaski et al. 2015). The viability percentage in this study showed a tendency to be higher in the lower fraction. The low percentage of viability in the upper fraction is caused by damage to the plasma membrane due to centrifugation, resulting in the death of spermatozoa, which are able to absorb the eosin-nigrosin dye used (Samplaski et al. 2015). Parera and Lenda (2023) add that metabolic processes will occur during spermatozoa storage. Spermatozoa will produce a byproduct in the form of lactic acid, which causes changes in pH in the surrounding medium, which, over time, will kill spermatozoa.

The results obtained in this study showed a lower percentage of abnormalities than research previously reported by Teken et al. (2020), that the abnormality of sexing spermatozoa in the upper fraction was 18.78%. In the lower fraction, it was 14.76%. This percentage is considered suitable for AI because it is still on standard of less than 20%. Evaluation of abnormalities is important in assessing the quality of spermatozoa because cells that experience abnormalities will disrupt the fertilization process, which will impact implantation and pregnancy rates in livestock (Putra et al. 2019).

The percentage of intact plasma membrane in this study showed a tendency to be higher in the lower fraction. The low percentage of the intact plasma membrane in the upper fraction is possibly caused by the process of sexing and freezing of spermatozoa, which causes physical injury, cold shock, and the formation of reactive oxygen species (ROS) which can reduce spermatozoa metabolism. ROS can induce spermatozoa membrane peroxidation, which will result in a decrease in the percentage of membrane integrity (Aghazarian et al. 2019).

Kinematics measurement of spermatozoa by the CASA system refers to the movement characteristics of spermatozoa (Aghazarian et al. 2019). This assessment is able to predict DNA damage related to male fertility (Valverde et al. 2019). The low percentage of spermatozoa quality in this study is thought to be caused by the sexing process with a relatively long incubation time, around 45 minutes. Incubation of spermatozoa during the sexing process can influence the success of the sexing process. A short time will produce a proportion of spermatozoa with X and Y chromosomes, but over a long time, it will cause an increase in spermatozoa damage (Anwar et al. 2019). The washing and centrifugation stages of spermatozoa in the sexing process have the potential to reduce the quality of spermatozoa (Luzardin et al. 2020). In addition, centrifugation will cause the release of ROS by dead spermatozoa (Katigbak et al. 2022), which are formed from mitochondria and plasma membranes and, in large quantities, will cause oxidative stress. Oxidative stress will interact with DNA, lipid, and protein molecules, causing DNA fragmentation, lipid peroxidation, axonemal damage, superoxide formation, and enzyme denaturation (Sabeti et al. 2016).

The results of this study showed that the percentage of acrosome integrity in the upper fraction and lower fraction was around 71.74-74.79% and 83.9-87.45%, respectively. The low percentage of acrosome integrity in the upper fraction is thought to be due to physical injury, cold stimulation and oxidative stress, which can occur due to the sexing process. It can cause a decrease in energy metabolism and spermatozoa mitochondrial processes (Sun et al. 2020). Lipid peroxidation due to oxidative stress will result in damage to the acrosome and plasma membrane, resulting in decreased metabolism and damage to the function and morphology of spermatozoa (Parera and Lenda 2023).

The percentage of DNA fragmentation in this study in the upper fraction was around 97-98.25% and 98.46-99.02% in the lower fraction. DNA fragmentation of spermatozoa is caused by damage to the plasma membrane (Palacin et al. 2020). Katigbak et al. (2022) added that centrifugation can cause a decrease in the biochemical and DNA integrity of spermatozoa due to contact between spermatozoa cells and ROS released by dead spermatozoa cells during centrifugation.

On the SDS Page results of polled Bali bull were found several proteins with different molecular weights, namely HSP70, TUBA8, Phospholipase C and Zeta, SP17 and BSFP (Fig. 1). The results showed that these proteins were positively correlated with motility, viability, abnormalities, and intact plasma membranes but negatively correlated with acrosome integrity and DNA fragmentation (Fig. 2). Evaluation of spermatozoa proteins correlates with motility (Cao et al. 2018) and can be a biomarker in predicting male fertility (Agarwal et al. 2020).

The identified protein plays a role in maintaining motility, viability, abnormalities, and IPM with a molecular weight of 70kDa. Heat Shock Protein 70 is a identified protein with a molecular weight of 70kDa (Naaby-Hansen and Herr 2010). The results showed that there was no

HSP70 protein in the upper fraction of males 11437 and 11746, so the quality is low. This indicated the critical role of the HSP70 protein in maintaining membrane integrity so that cell metabolic processes are maintained. Low expression of HSP70 will reduce sperm quality (Zhang et al. 2015) due to damage to the plasma membrane (Naaby-Hansen and Herr 2010). Outer dense fiber (ODF) protein is also involved in modulating spermatozoa motility and plasma membrane by protecting sperm tail shear forces in mammals with molecular weight variations of 70.8kDa (ODF 2/1) and 73.4 kDa (ODF 2/2) (Petersen et al. 1999).

In this study, there was also a positive correlation between motility and the TUBA8 protein (r=0.087). Bhagwat et al. (2014) reported that the TUBA8 protein is related to spermatozoa motility. The TUBA8 protein, also known as tubulin alpha 8, is associated with spermatogenesis, specifically in the development of spermatids as well as in controlling the initial formation of the acrosome. The TUBA8 protein was identified with a molecular weight of 50kDa (Serrano et al. 2022).

The results showed a positive correlation between sperm motility and a molecular weight of 33kDa, which was identified as the Phospholipase C zeta protein (PLCζ). This protein was only found in male 11746 in the upper and lower fractions, which were able to maintain motility, DNA integrity and acrosome reaction, although it was not positively correlated with acrosome reaction and DNA integrity. PLCζ with a molecular weight of 33-34kDa (Rosyada et al. 2020) is a protein found in sperm that produces calcium (Ca^{2+}) oscillations by mitochondria, which induce egg cell activation and early embryo development and it will be active in the oocyte during the fertilization process (Saleh et al. 2020). Storage and release of Ca2+ signals are carried out by mitochondria (Costello et al. 2013). Sperm mitochondria play a role in ROS production, calcium signaling, and apoptosis, as well as being a prerequisite for motility, acrosome reaction, and DNA integrity (Durairajanayagam et al. 2021).

Sperm protein 17 (SP17) with a molecular weight of 22-24kDa (Wen et al. 1999) plays a role in the fertilization process by regulating capacity, sperm maturation, acrosome reaction, and interactions between sperm and the zona pellucida of the oocyte (Chiriva-Internati et al. 2009). Teixeira et al. (2006) reported that a protein with a molecular weight of 12kDa, known as the BSFP protein (Buck Seminal Fluid Protein), was found in seminal plasma and also in the spermatozoa in this study. The results of the SDS page of protein bands between fractions to separate X and Y sperm can be an additional reference in producing sexing sperm. To optimize the results, it can be further tested with DNA validation tests or protein profiling based on molecular size, which still needs further investigation.

Conclusion

The decrease in spermatozoa quality was thought to be caused by physical injury to spermatozoa due to the sexing process. The protein bands of X and Y spermatozoa of polled Bali bulls were positively correlated with motility, viability, abnormalities and IPM, but negatively correlated with acrosome integrity and DNA fragmentation.

Author's Contribution: Conceptualization: Hasbi Hasbi, Sudirman Baco and Ekayanti Mulyawati Kaiin; Data curation: Andi Tifal Nurgina, and Erni Damayanti; Investigation: Andi Tifal Nurgina, Hasbi Hasbi, Ekayanti Mulyawati Kaiin, Tulus Maulana, Muhammad Gunawan, and Erni Damayanti; Methodology: Andi Tifal Nurgina, Hasbi Hasbi, Ekayanti Mulyawati Kaiin, Tulus Maulana; Resources: Andi Tifal Nurgina, Hasbi Hasbi, Ekayanti Mulyawati Kaiin, Tulus Maulana, Muhammad Gunawan, Erni Damayanti; Supervision: Hasbi Hasbi, Sudirman Baco, Ekayanti Mulyawati Kaiin, Ratmawati Malaka, Sri Gustina; Writing – original draft: Andi Tifal Nurgina; Writing – review & editing: Hasbi Hasbi, Sudirman Baco, Ekayanti Mulyawati Kaiin, Ratmawati Malaka, Sri Gustina, and Erni Damayanti.

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